ORIGINAL ARTICLE

The Effect of Co-Metabolism on Removal of Hexadecane by Microbial Consortium from Soil in a Slurry Sequencing Batch Reactor

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Abstract

Background: Among Alkanes, N-Alkanes with medium chain have been identified as the most important contaminants of the soil. N-hexadecane ($C_{16}H_{34}$) with low solubility in water also belongs to this group and has been used by many researchers as a model contaminant. The present study aimed to investigate the effect of the external source of carbon (glucose) as co-substrate on removal of hexadecane from the soil.

Methods: In this study, a Slurry Sequencing Batch Reactor (SSBR) was used as a pilot by a bacterial consortium, including bacterium *Acinetobacter radioresistens, Bacillus subtilis, and Pseudomonas aeruginosa*, in order to remove different concentrations of hexadecane (1,4,7, and 10 percent).Sampling was performed four times during the sedimentation step. Then, the samples were analyzed by GC-FID and the results were analyzed statistically.

Results: The results showed that hexadecane removal (%) by the microbial consortium was higher in lower initial concentrations in such a way that the biological removal of hexadecane was respectively 45.95%, 38.55%, 34.39%, and 32.40% in the concentrations of 1%, 4%, 7%, and 10% on the third day. Moreover, adding the external carbon source (glucose) on the first day caused a 16% increase in hexadecane removal, which is 1.4 times more than the amount of hexadecane removal in the conditions without co-metabolism.

Conclusion: The results showed that SSBR could be used as an exit-situation effective method for hexadecane removal in low concentrations through considering the effective factors in its function, such as dissolved oxygen, pH, and temperature. Also, adding the secondary carbon source could be effective in hexadecane removal from the soil. Yet, this effect might vary on different days.

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Keywords: Soil, Hexadecane, Slurry Sequencing Batch Reactor (SSBR), Microbial Consortium, Co-metabolism

Introduction

In general, considerable amounts of toxic and refractory materials entered ourenvironment by human activities. Among these materials, hydrocarbons are accumulated in aquatic and soil ecosystems with different degrees of biological availability.[1] Sometimes, the amount of pollution is very high and even in some cases, the total concentration of hydrocarbons reaches above 450000 mg kg^{-1}.[2] Aliphatic hydrocarbons with small linear chains (C_{16}-C_{32}), carbon sources, and their energy are decreased easily by a number of microorganisms.[3] Oil

products, such as diesel fuel, heavy oil, fuel residuals, and mineral oil are the common contaminants of the soil. Hexadecane has molecular weight, density, melting point, boiling point and vapor pressure of 266.44 g mol^{-1}, 0.733 g cm^{-3} (at 25 °C), 18 °C , 287 °C , 100 pa (at 105.3 ˚C) , respectively.[4]

Different physical, chemical, combustion, and biological methods can be used for treatment of oil contaminated soil.Washing the soil is among the physical methods for treatment of oil contaminated soils.[5] The US Environmental Protection Agency (USEPA) has defined soil washing as a treatment technology for oil-polluted soils.[6] Soil washing is faster than other treatment methods, such as biodegradation, chemical treatment, and combustion.[7, 8] This process can be used for oil removal in large particles, such as sand and gravel, but removal of oil from small particles, such as silt (5-7.5 µm) and clay (<5 µm), is difficult. Oil products are concentrated on the surface of small particles due to the high specific surface area.[8] If washing method is used, the soil including small particles should be landfilled. Overall, 20-50% of most soils have been formed by small particles[7-9] and in case these soils are used for the second time, they should be refined more after being washed. As a whole, change of phase is done in non-biological methods and the contaminant enters an environment from another one while the problem hasnot been solved yet.[10] In biological methods, on the other hand, the contaminant is destroyed. Nonetheless, the problem of biological methods is the low rate of degradation.[11] Hassanshahian and colleagues isolated 8 strains of alkane degrading bacteria in petroleum reservoir wastewater of Tehran and Kerman Provinces of Iran. To determine the effect of various concentrations of hexadecane on bacterial growth, fifteen alkane degrading bacteria were isolated and 8 strains were selected as powerful degradative bacteria. These 8 strains are related to *Rhodococcus jostii, Stenotrophomonas maltophilia, Achromobacter piechaudii, Tsukamurella tyrosinosolvens, Pseudomonas fluorescens, Rhodococcus erythropolis, Stenotrophomonas maltophilia, Pseudomonas aeruginosa genera*. The isolated bacteria were grown in different concentrations of hexadecane (1%, 2.5%, 4%, 5.5% and 7%); then, optical density was read at 600 nm every day for each strain that was incubated at 30 ˚C. All bacterial strains were grown in hexadecane (1%) for one week with shaking (160 rpm). During this week, everyday the optical density was read for each strain until the end of the week and O.D that was related to exponential phase was reported as growth rate. After one week, hexadecane biodegradation was analyzed by GC-FID method. The results showed that all the strains could degrade up to 50% of hexadecane after one week of incubation. The best concentration of hexadecane that allowed the high growth rate in all bacterial strains was 2.5%.[12]

Compared to other biodegradation methods, Slurry Sequencing Batch Reactor (SSBR) has a higher degradation rate of oil materials.[13] Cassidy and colleagues notified that the concentration of diesel oil in the soil (sandy loamy) decreased from 12 g kg^{-1} to lower than 1 g kg^{-1} by SSBR after adjustment of dissolved oxygen and pH.[14] If SSBR process is used for treatment of fine particles, soil washing and use of SSBR process afterwards can be a suitable method for cleaning such soils. However, in SSBR, the biodegradation rate of fine particles is lower than that of coarse particles.[15] Geerdink and colleagues reported that the concentration of diesel oil in the soil with high silt and clay decreased from 17 to 0.5 g kg^{-1} in SSBR after 10 weeks.[16] Biodegradation of oil products in the soil is time consuming. Therefore, these technologies must be amended to accelerate bioremediation of contaminated fine soils. Surfactant could accelerate the removal of oil compounds from soils by increasing bioavailability.[10] Nano and colleagues increased the removal rate of these materials by adding a surfactant to the soil polluted with diesel oil.[17] Microorganisms can produce bio-surfactant,thereby increasing the biodegradation rate.[18] Khezri and colleagues investigated the effect of SSBR on biodegradation of soils polluted with Total Petroleum Hydrocarbons (TPH). These results showed that 95% of TPH with the initial concentration of 67500 mg kg^{-1} was removed by the SSBR after 90 days.[19] In addition, Hasanlou and colleagues used the SSBR for removal of TPH and Polycyclic Aromatic Hydrocarbons (PAH). The results showed that 96% of TPH and 100% of PAH were removed at the end of each cycle.[20]

Co-metabolism was introduced by Wilson in 1985. Afterwards, it was reintroduced by McCarty in 1987. Co-metabolism is a process in which a contaminant is degraded with an enzyme or a co-factor produced during the microbial metabolism of another compound. Co-metabolism only stimulates indigenous bacteria which are capable of degrading the contaminants and co-substrates (methane, propane, toluene, etc.). Co-metabolism has been used for more than 20 years for a number of resistant contaminants, such as Polychloroethylene, Trichloroethylene, T.N.T, Dioxin, Atrazine, Aromatic Hydrocarbons, Chlorinated Alkanes, and Aliphatic Halogens. Nonetheless, the essential nutrients are not completely available in most systems and, consequently, biodegradation is limited.[21]

In many studies, the alkane degrading bacteria were isolated from different environments, such as wastewater and oil-contaminated soils.[22, 23] Jurelevicius and colleagues isolated the alkane degrading bacteria from the George King island soils and found that a high variety of alkane degrading bacteria existed in this

environment.[24] Quatrini and colleagues also isolated a number of N-alkane degrading bacteria from the Mediterranean shoreline.[25] In a study by Plangklang and Reungsang, the effectiveness of bioremediation technology in removal of carbofuran from contaminated soil using a bio-slurry phase sequencing batch reactor (SBR) was investigated. A 2-L laboratory glass bottle was used as a bioreactor with a working volume of 1.5 L at room temperature (27±2˚C). One total cycle period of the SBR was comprised of 1 h of fill phase, 82 h of react phase, and 1 h of decant phase. The carbofuran concentration in the soil was 20 $mgkg^{-1}$ soil. A carbofuran degrader isolated from carbofuran phytoremediated soil, namely Burkholderia cepacia PCL3 (PCL3) immobilized on corncob, was used as the inoculum. The results revealed that bioaugmentation treatment (addition of PCL3) gave the highest percentage of carbofuran removal (96.97%), followed by bioaugmentation together with biostimulation (addition of molasses) treatment (88.23%).[26]

Overall, the biological treatment process has abundant advantages compared to other methods for removal of oil pollution from soils. On the other hand, biological reactions can be accelerated in a slurry system due to the increased contact between the contaminant, nutrients, and microorganisms. Considering the significance and global expansion of soil and water pollution with petroleum, numerous studies are necessary to be conducted. Therefore,the present study aimed to investigatethe effects of different factors,such as dissolved oxygen, pH, temperature, and glucose (co-metabolant) on removal of hexadecane in an SSBR. In this study, a bacterial consortium, including *Acinetobacter radioresistens, Bacillus subtilis, and Pseudomonas aeruginosa,* was used.

Materials and Methods

Materials Specifications

Chemical materials used in this study included Hexadecane, 1,2,4-trchlorobenzene, acetone, glucose, HCl, H_2SO_4, NaOH, NaN_3, Na_2SO_4, NH_4Cl, NaCl, $MgSO_4$, $FeCl_3.6H_2O$, $CaCl_2$ and $MnCl_2.4\ H_2O$. All chemical materials used in this study had 99.7% purity and were purchased from Merck, Germany.

Preparing the Soil

The soil used in this study was agricultural soil collected from Paskoohak region ,40 Km from Shiraz, Iran. The physicochemical analysis of the soil is presented in Table 1. In order to prepare the soil, first, the soil was sieved with a 10 mesh (2 mm) sieve for screening the soil and reaching uniformity. Then, it was soaked with distilled water and autoclaved for 15 minutes at 121˚C. At the end, it was located in the oven at 160˚C to make it sterile and dry and reach its primary weight. After being dried, it was sieved with a 10-mesh (2 mm) sieve. At the end, it was transferred to a 1-liter container and contaminated in different concentrations. In order to artificially contaminate the soil with hexadecane at 1, 4, 7, and 10% concentrations, first the necessary amount of hexadecane was dissolved in 30 ml hexane. Then, the obtained solution was added to the soil. In order for uniform distribution of hexadecane in the soil, the soil was completely submerged in the solution. Then, the soil was regularly mixed in short time intervals and it was permitted to dry completely under the vent at room's temperature. At the end,a one-week period was considered for absorption of hexadecane by the soil.

Preparing the Essential Nutrients

In order for the SSBR to operate, in addition to contaminating the soil with hexadecane, essential nutrients and water were also needed for the microorganisms. In this study, tap water was used to prepare the essential nutrients. The specifications of tap water were measured using polarography. The nutrients included 2.5 g L^{-1} NH_4Cl,0.5g L^{-1}NaCl, 0.3 g $L^{-1}MgSO_4$, 0.3 g $L^{-1}Fecl_3.6H_2O$, 0.01g $L^{-1}CaCl_2$, and 0.01 g L^{-1} $MnCl_2.4\ H_2O$. Then, pH was adjusted to 7±0.5. All the media were autoclaved at 121˚C[22] for 15 minutes and then added to the SSBR.

Preparing the Solid Mineral Medium

In order to keep the used bacteria fresh in the bioreactor,they were cultured weekly on the mineral

Table 1: Results of the physicochemical analysis of the soil used for contamination

Properties	Amounts	Properties	Amounts
pH of reaction	7.58	**EC (Ms/cm*)**	1.21
Humidity (%)	50	**Lime (%)**	44.85
Organic carbon (%)	0.93	**K (ppm)**	186
P (ppm)	10.1	**Zn (ppm)**	12
Fe (ppm)	10.2	**Cu (ppm)**	1.1
Soil texture	Loam	**Mn (ppm)**	20.4
Sand (%)	33.6	**Silt (%)**	46.4
Clay (%)	20	**N_2**	0.09

*milisimenes per centimeter

medium including hexadecane as the only source of carbon. In order to create this medium, first 1 g L^{-1} yeast extract was added to the bottle. Afterwards, 15gr Agar-Agar was added and pH was adjusted at 7±0.4.Then, the media were added to the plates and kept to become solid. In order to provide the source of carbon, 20 µl hexadecane was poured on the plates and it was distributed evenly by the pipette insuch a way that a very thin layer of hexadecane was located on the surface of the plates. The plates were then located in the incubator (37.5˚C, 24 hours) in order to grow the bacteria.

Preparing and Culturing the Bacteria in the Nutrient Broth Medium

In this study, a microbial consortium including three kinds of bacteria,i.e. *Acinetobacter radioresistens, Bacillus subtilis, and Pseudomonas aeruginosa,* were used.These bacteria were isolated from the soil in another study.[22] In order to increase the number of bacteria and add them to the reactor, the bacteria which had been cultured in the Agar-Agar medium were cultured in the nutrient broth medium. Afterwards, they were located on the mixer in the incubator at 37.5˚C for 24 hours in order to be grown. There after, the nutrient broth medium including the grown bacteria was transferred to the test tubes in order to be isolated completely and then centrifuged at 4000 rpm for 5 minutes. Finally, the optical density of the bacteria was measured at the wave length of 600 nm to ascertain the uniformity and equal distribution of the bacteria in all the bioreactors. It should be noted that the optical concentration of the bacteria reached one, using normal saline. After all, the bacteria were added to the reactor.[11]

Measuring the Number of Active Bacteria

To determine the number of the bacteria grown in the SSBR, the samples were taken from the reactor at different times of operation and then cultured at three dilutions,i.e. 10^{-1}, 10^{-2}, and 10^{-3}, on the nutrient agar medium. After that, the samples were located in the incubator at 37.5˚C for 24 hours. After being assured about their growth, the colonies were counted by the colony counter and the number of bacteria was reported based on CFU/ml.

Hexadecane Extraction and Analysis

In order to analyze the residual of hexadecane from the soil, it was extracted from the soil through USEPA method 3550c.[27] Briefly, the sample was taken from the deposited sediment after the process of sedimentation and dried at 37.5˚C. Then, 0.5gr of the dry soil was mixed with 0.5gr anhydrous sodium sulfate as the dehumidifier factor. The content of the volumetric flask reached 5 ml with 4ml normal hexane and it was completely mixed in order to mix the soil with normal hexane. The volumetric flask was then put in the ultrasonic bath at 30˚C for 2 minutes in order to extract hexadecane. At the end of the extraction time, the upper liquid of the volumetric flask was transferred to a test tube. In order to extract hexadecane more efficiently, this operation was repeated twice. After that, the test tube was located in the centrifuge system at 4000 rpm for 5 minutes to isolate the soil and upper liquid completely. Then, 1ml of the upper liquid was taken by the sampler and then moved to the vial. Afterwards, 10 µl of the internal standard (1-2-4, trichlorobenzene) was added to the vial by Hamilton syringe. Finally, 2 µl was taken from the vial content by the injection syringe and injected to the GC-FID system. The recovery percentage of hexadecane was averagely obtained as 72% at different concentrations through the extraction method.

In order to measure the residual of hexadecane in the study samples, the GC-FID system was used. CP-SILSCB (silica, USA) column (30 m length×0.025 mm id×0.25 µm film thickness) was used at a temperature program of 80°C for 1 min, increased to 125°C at 10°C min^{-1}, held at 125°C for 5 min, increased to 270°C at 40°C min^{-1}, and held at 270°C for 4 min. Moreover, nitrogen was used as a carrier gas at a constant flow of 2.7 ml min^{-1}. Injector and detector temperatures were 210 and 250°C, respectively.In addition, the detection limit of the gas chromatography system was 166.5 mg kg^{-1} for hexadecane.[11]

Operation of SSBR

In a glass reactor with a working volume of 11 L containing polluted soil, the nutrients and bacteria were mixed with enough tap water to bring the total slurry volume to 5 L (Figure 1). Tap water was used for making the slurry of urban water. Each cycle of the reactor lasted for 74 hours, including 1 hour filling, 72 hours reaction and sedimentation, and 1 hour discharge. After the primary preparation of the reactor, tap water, bacterial inoculation liquid, 250 gr polluted soil, and the essential nutrients were added. The first sample was taken one hour after the beginning of the reactor's working as the sample of the zero days. This one hour was considered for being assured about the uniformity of the materials in the reactor. In each cycle, the reaction process lasted for 21 hours. Then,the reactor was located in the depositing condition for 3 hours and the sample of the first day was taken. This was performed for three days (one cycle). At different times, 8 ml aliquots were sampled to determine the residual amounts of hexadecane. Since the pressure of hexadecane steam is 100 Pa at 105.3˚C, the samples of the deposited sediment were located in the incubator at 37.5˚C in

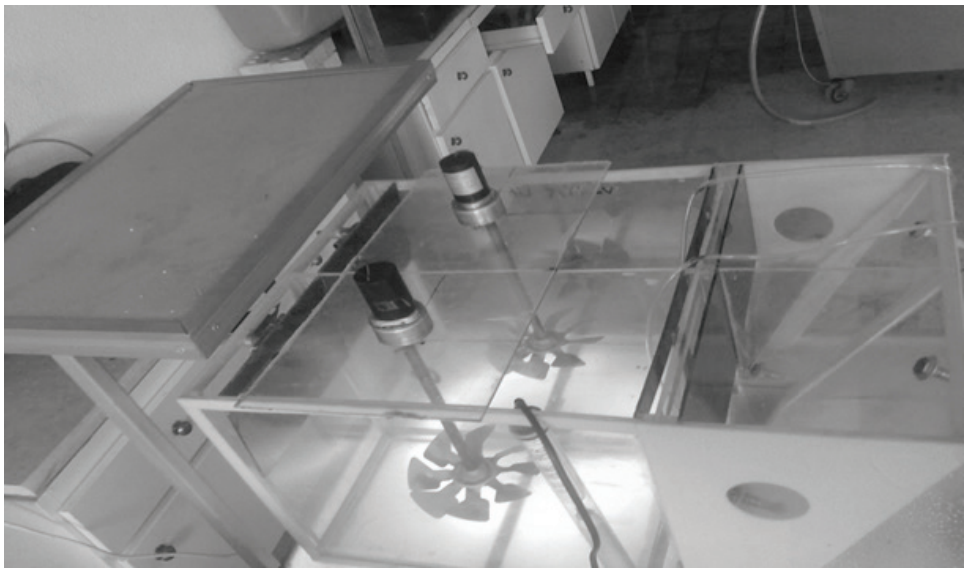

Figure 1: This figure shows the Slurry Sequencing Batch Reactor (SSBR).

order to be dried. A blank reactor was also operated along with the main reactor simultaneously. This reactor had all the conditions of the main reactor, except for the bacteria. To ascertain the lack of bacteria in the blank reactor,after extracting and injecting with chromatography system, the removal of hexadecane was determined in the blank reactor at all concentrations. The biodegradation rate of hexadecane (%) was obtained by subtracting the total removal rate of hexadecane (%) at all concentrations in the main reactor from the total removal rate of hexadecane (%) in the blank reactor. Furthermore, to determine the total removal rate of hexadecane (%) in the main and blank reactors, each reactor was operated for four times at 1, 4, 7, and 10 percent concentrations. During this time, other operation parameters, such as dissolved oxygen, pH, bacteria count, and temperature, were monitored. Dissolved oxygen was measured using a DO meter (HACH-cat. no.58258-00), pH was measured using a pH meter (pH lab-metrohm, Swiss 827), and the temperature was measured using thermometer. The method used for measurement of the number of active bacteria was explained in section 2-5. The results showed that the highest biodegradation rate of hexadecane occurred at the concentration of 1% hexadecane. Thus, the reactor was prepared in similar conditions to the previous part and then 10 gr (2 percent) glucose was added to the reactor as the secondary source of carbon in order to compare the effect of adding an external source of carbon (glucose) on hexadecane removal (%) in the conditions with and without co-metabolism at the concentration of 1% hexadecane. A blank reactor was also considered for concentration of 1%, and the samples were taken from the blank reactor on all the experiment days. After extracting the samples, injecting them to the chromatography system, and analyzing the results, the total removal percentage of hexadecane in the main and blank reactors was determined at the concentration of 1% and adding of the external source of carbon (glucose). The biodegradation rate of hexadecane (%) was obtained by subtracting the total removal rate of hexadecane (%) at the concentration of 1% in the main reactor from the total removal rate of hexadecane (%) in the blank reactor. Then, the obtained biodegradation rates were compared.

Statistical Analysis

Various statistical methods are available for optimizing the removal conditions. In this study,one factor at a time approach was used.[28] All the samples were taken in duplicates and the line in the graphs represents the average value. All the data obtained in the study were subjected to statistical analysis of correlation cofficient with SPSS,version 19.0. The significance level was considered to be $P<0.05$ probability level.

The Study Date and Place

The current research was conducted in the first half of 2014. Also, All experiments were conducted as a pilot at laboratory.

Results

In this study, a microbial consortium including three kinds of bacteria,i.e. *Acinetobacter radioresistens, Bacillus subtilis, and Pseudomonas aeruginosa*, was used. Gram staining of bacterial consortia is shown in Figure 2.

The water added to the reactor was tap water the specifications of which were measured using the polarography system as follows:(Based on the mgL^{-1})

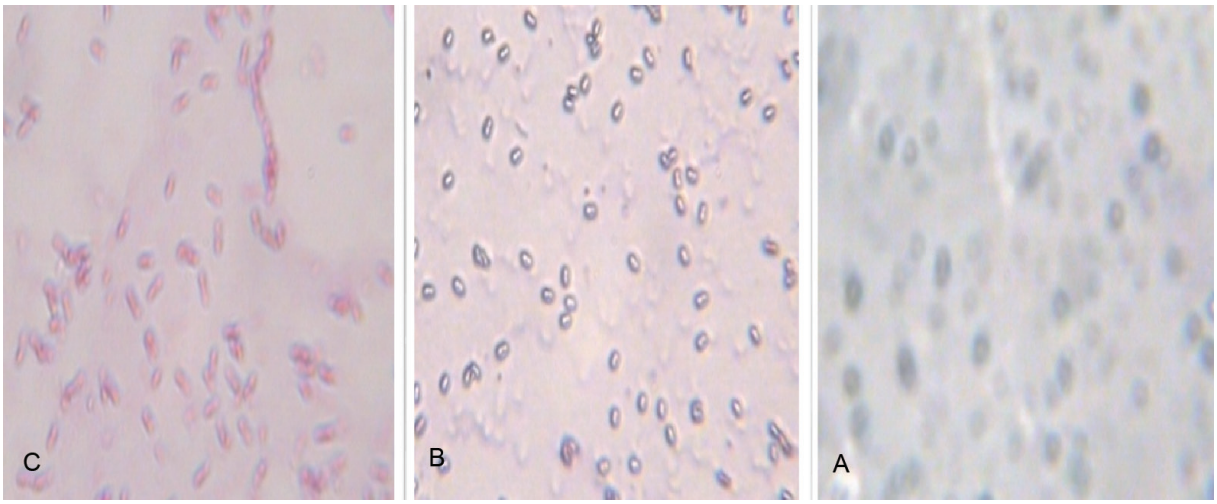

Figure 2: These figures show the gram staining of bacterial consortia.(a) *Acinetobacter radioresistens,* (b) *Bacillus subtilis,* (c) *Pseudomonas aeruginosa.*

nickel: 1-10, cobalt: 30-60, solfate:120-160, nitrate: 25-37, nitrite: 2-8, chloride:120-140, bromide: 1-2, floride: 0.4-1, total iron: 0.07, iron: 0.04, calcium: 60, and magnesium: 80-120. The soil used in the study was also analyzed using a physicochemical method and the results are shown in Table 1.

In order to determine the biodegradation percentage of hexadecane in the SSBR at the concentrations of 1,4,7, and 10 percent, the samples were collected on the zero (one hour after the reactor's beginning of working), first, second, and third days. After extraction, the samples were injected into the gas chromatography system. Then, the results were statistically analyzed and the biodegradation percentage of hexadecane was determined. The results arepresented in Figure 3.

In order to determine the growth rate of bacteria in different concentrations during different days, some samples were taken from the reactor. The results related to the number of bacteria (CFU/ml) in different experimental conditions during different days are shown in Figure 4.

The samples were also collected to determine the amount of dissolved oxygen in different concentrations of hexadecane during different days. The results related to the dissolved oxygen (as mgl^{-1}) in different experimental conditions during different days are shown in Figure 5. Accordingly, a negative, strong linear relationship was observed between hexadecane concentration and amount of dissolved oxygen on the zero, first,second, and third days ($r_{=}$-0.95, P=0.045) insuch a way that increase of concentration led to a decrease in the amount of dissolved oxygen.

In order to determine the pH, some samples were taken from the reactor on the zero (one hour after the reactor's beginning of working) first, second, and third days. pH was measured using the pH-meter system. The results showed that in concentrations of 1,4,7, and 10 percent of hexadecane,pH respectively decreased to 0.73, 0.75,0.77, and 0.83 after three days. Moreover, a significant strong, negative, linear relationship was found between hexadecane concentration and pH

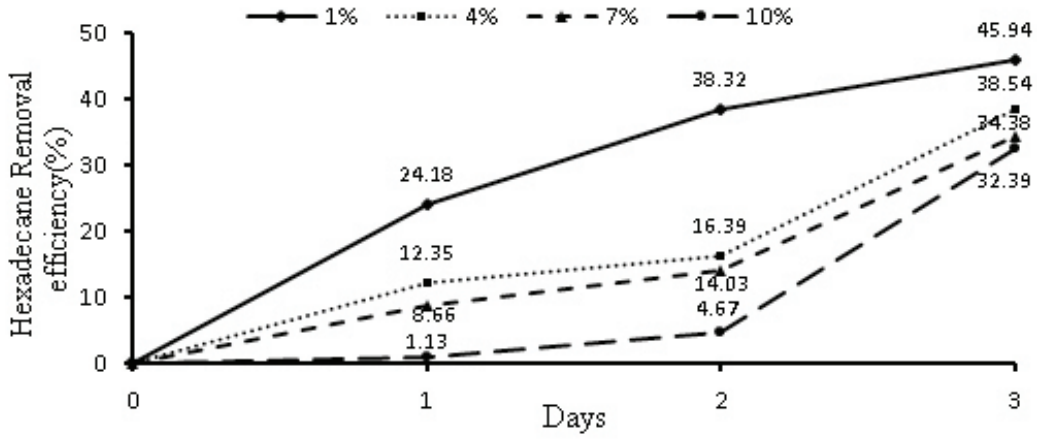

Figure 3: This figure shows the biodegradation percentage of hexadecane at the concentrations of 1,4, 7, and 10 percent in the SSBR.

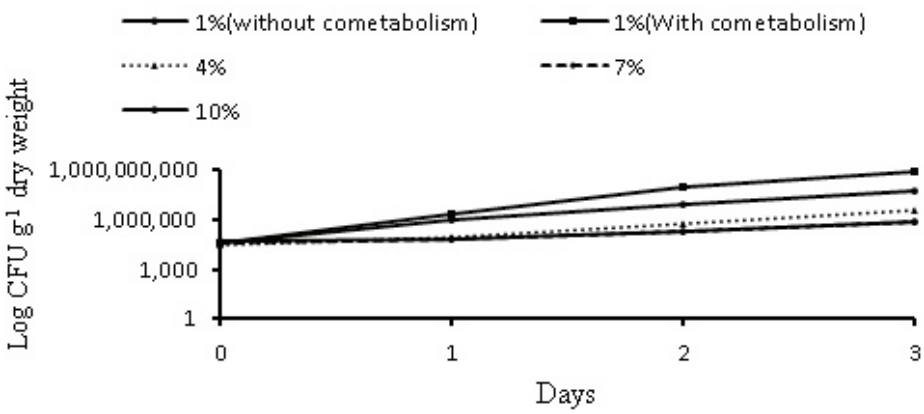

Figure 4: This figure shows the evolution of the cultivable bacteria population (Hexadecane concentrations of 1, 4, 7, and 10 percent and hexadecane concentration of 1% along with co-metabolism in the SSBR).

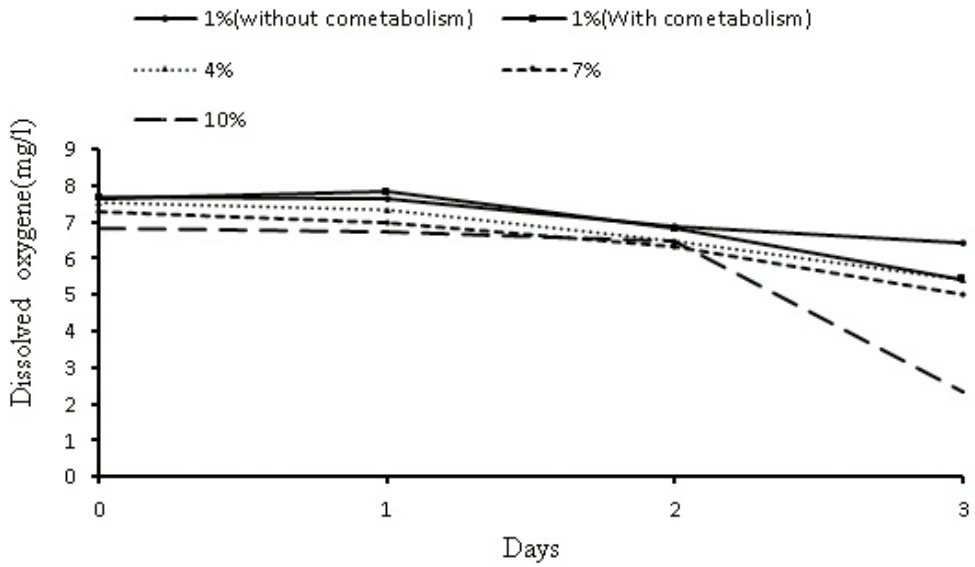

Figure 5: This figure shows the amount of dissolved oxygen at the concentrations of 1, 4, 7, and 10 percent of hexadecane and concentration of 1 percent of hexadecane along with co-metabolism in the main SSBR.

on the zero, first, second, and third days ($r_{=}$0.96, P=0.037) insuch a way that pH decreased following the increase in the concentration.

Also, the samples were collected from the reactor on the zero (one hour after the reactor's beginning of work), first, second, and third daysin order to determine the temperature. Based on the results, in concentrations of 1, 4, 7, and 10 percent of hexadecane, temperature respectively increased to 4.5, 7, 7.2, and 7.4 after three days. Furthermore, a significant, strong, positive, linear relationship was found between the concentration and temperature on the zero, first, second, and third days ($r_{=}$0.92, P=0.035) in such a way that increase of concentration resulted in an increase in the temperature.

Since the highest percentage of hexadecane biodegradation was related to the concentration of 1 percent, this concentration was used to investigate the effect of co-metabolism. Samples were taken from the reactor in order to determine the effect of adding an external carbon source (glucose) on the biodegradation percentage of hexadecane at the concentration of 1% and compare the results to the condition without co-metabolism on the zero (one hour after the reactor's beginning of working), first, second, and third days. After extraction, the samples were injected to the gas chromatography system. Then, the results were statistically analyzed and the biodegradation percentage of hexadecane was determined in the condition with the effect of co-metabolism. Afterwards, the results were compared with the condition without co-metabolism and the findings are presented in Figure 6.

In this study, in order to measure the residual of hexadecane in the study samples, the GC-FID system was used. The chromatogram of hexadecane and internal standard (1, 2, 4.trichlorobenzene) is shown in Figure 7.

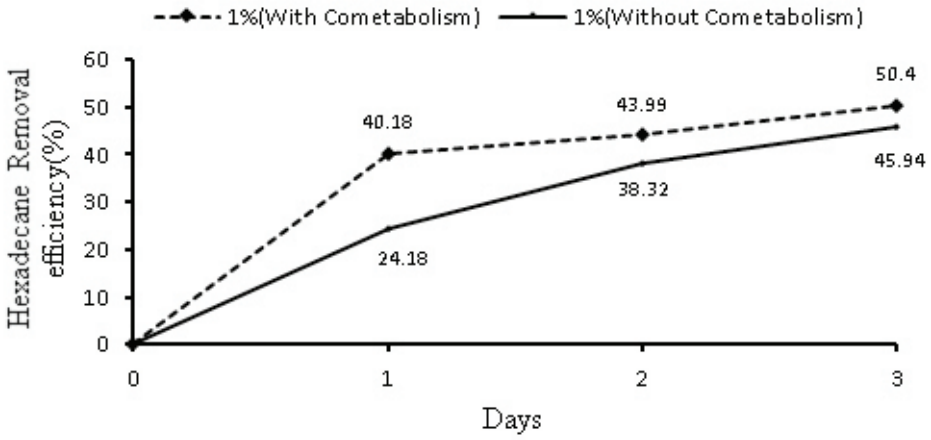

Figure 6: This figure shows the comparison of the removal percentage of hexadecane at the concentration of 1% hexadecane in two conditions with and without co-metabolism (glucose) in the SSBR.

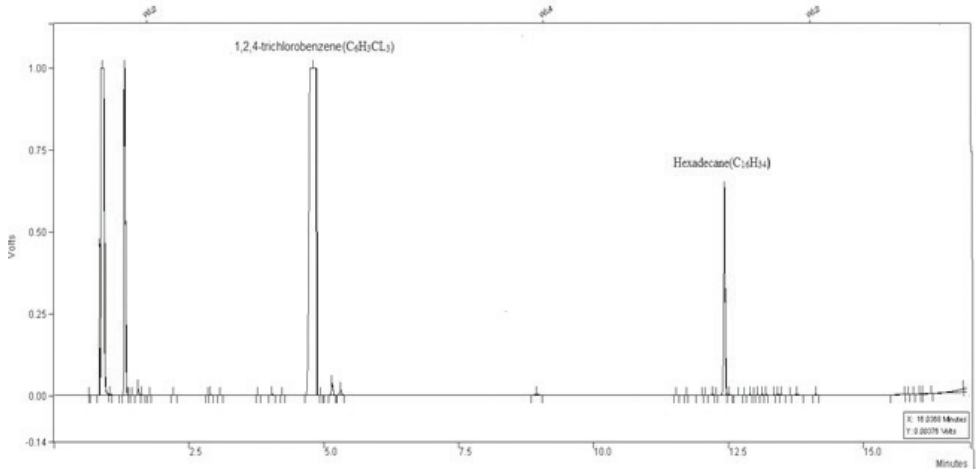

Figure 7: This figure shows the GC-FID chromatogram of hexadecane and 1, 2, 4-trichlorobenzene.

Discussion

In the percent study, a bacterial consortium, including *Acinetobacter radioresistens*, *Bacillus subtilis,* and *Pseudomonas aeruginosa,* was used to remove hexadecane in an SSBR.These bacteria were previously isolated and recognized by Samaei and colleagues from the soil contaminated with oil in Abadan refinery and agricultural soil of Darab.[29] Compared to other biodegradation methods, Slurry Sequencing Batch Reactor (SSBR) has a higher degradation rate of oil materials.[13] SSBR has been tested in bench and pilot scales with different operational conditions. This reactor provides one of the most common and the best operational conditions. Hexadecane is a refectory contaminant in the environment. The low solubility of this hydrocarbon contaminant in water causes it to be unavailable for the microorganism.[12] Solubility of hexadecane in water is lower than $0.9\times10^{-4}mgl^{-1}$.[30] Therefore, it is supposed that bacteria should have a number of mechanisms for absorbing and using this hydrophobic substrate. The cell surface hydrophobicity and the production of emulsifiers may be two mechanisms for better degradation and absorbance of hexadecane in a liquid medium. Hassanshahian and colleagues found a direct relationship between the surface hydrophobic of cell and emulsion activity and biodegradation.[31] Thus, when a bacterial strain has a higher surface hydrophobicity, it can produce more emulsifier, eventually increasing the biodegradation. Alkanes are catalyzed byoxygenizes. This enzyme plays an important role in biodegradation and co-metabolism degradation of Alkanes. In the present study, an aerating pump and a mixer were used in the reactor for creation of aerobic conditions and degradation of hexadecane by oxygenize enzyme. Biodegradation is based on the use of the microbial population which has the ability of degradation of special contaminants. In the biodegradation process, the contaminants are completely mineralized.[32-34] Up to now, most of the investigations have studied the biodegradation of aliphatic hydrocarbon in concentrations lower than 1%.[25] In the present study, however, 1-10% concentrations were assessed. The results showed that hexadecane removal percentage was higher in lower concentrations compared to higher ones. Also, as time went by, the removal rate of hexadecane increased in all the concentrations (Figure 3). This

might be due to the growth of bacteria over time, higher adaptation of the bacteria with the existing conditions, and increase in the amount of produced biosurfactants. In other words, a larger amount of hexadecane changes to the emulsion over time that increases the bioavailability of hexadecane for the bacteria. Many studies have described production, specifications, and usage of biosurfactants as an active compound released by a microorganism.[35, 36] Moreover, Vasila-Tonkova and colleagues found that the degrading bacteria of oil have different adaptation mechanisms for using low solubility substrates and can produce biosurfactants which are able to increase the absorption rate of hydrocarbons.[37]

In the present study, the highest biodegradation percentage of hexadecane (45.95%) was observed at the concentration of 1% in a 3-day period (Figure 3). Hassanshahian and colleagues also investigated 8 bacterial strains at different concentrations of hexadecane (1-7%) and concluded that the concentration of hexadecane was decreased by 70% after one week of incubation at 32˚C.[31] In other studies, 40%[38] and 80% decrease of hexadecane[39] were observed after a 10-day period of incubation. In a study, Gomes and colleagues investigated microbial community dynamics in diesel waste biodegradation using sequencing batch bioreactor operation mode (SBR) in four cycles of 72 h; using optimized setpoints (pH, initial waste load, C:N ratio, aeration), they concluded that optimal conditions allowed the system to reach biodegradation of 53.3, 96.0, 76.2 and 75.0% at the end of cycles one, two three and four, respectively.[40] In the study by Venkata Mohan and colleagues, ex situ treatment of simulated pyrene-contaminated soil was studied in bio-slurry phase reactors operating in periodic discontinuous batch mode under anoxic–aerobic–anoxic–anoxic microe nvironment. Experiments were performed in six different bio-slurry phase reactors (retention time of 120 h; soil loading rate of 20 kg soil/m^3-day; operating temperature at 28±2˚C) by varying substrate concentrations(substrate loading rate (SLR), 0.12, 0.24 and 0.36 g pyrene/kg soil-day) and bioaugmentation application (domestic sewage inoculum; CFU-2×10^6). The performance of slurry phase reactors was found to be dependent on the applied SLR and application of bioaugmentation (domestic sewage as augmented inoculum). Control reactor (killed control) showed only 6% of pyrene degradation while the non-augmented reactor showed an efficiency of 34% (substrate degradation rate (SDR) 0.0165 g pyrene/kg soil-day). In the case of augmented reactors, the system operated with low SLR and showed a pyrene degradation efficiency of almost 90% (SDR-0.04 g pyrene/kg soil-day) and the reactor with high SLR showed 50% (SDR-0.025 g pyrene/kg soil-day) of pyrene degradation, indicating the dependence of performance on the substrate concentration. Colony forming units (CFUs) variation was in good agreement with the performance of the reactors with respect to pyrene degradation.[41]

In the current study, the highest bacterial growth was related to the concentration of 1% hexadecane (Figure 4). This implies that the bacteria are practically trapped in the oil layer through the increase of hexadecane and, consequently, their availability to the nutrients and dissolved oxygen is decreased and the C:N:P ratio is disrupted. Therefore, increase of hexadecane concentration reduced the number of grown bacteria. In the study by Hassanshahian and colleagues conducted on 8 bacterial strains in different concentrations of hexadecane, the growth rate of bacteria was more in lower compared to higher concentrations and the optimal concentration for bacterial growth was 2.5%.[31] In the study conducted by Gomes and colleagues, investigations of microbial diversity showed changes in the microbial community members at the end of the cycle one.[40] Li and colleagues, studying the dynamics of changes in microbial community structure during petroleum degradation, verified that the numbers of DGGE bands decreased from 40 to 25 when oil concentrations increased to 5,000 mg kg^{-1} of soil.[42] These results confirmed the selective pressure caused by the presence of recalcitrant compounds. Previous studies have shown pH as the main effective factor in the structure of microbial communities in the soils.[43, 44] Fierer and colleagues found a strong relationship between pH and moisture reduction, organic carbon, and C:N ratio of the soil.[43] Similarly, Patovinia and colleagues concluded that hexadecane concentration and pH were highly effective in biodegradation of hexadecane by bacteria.[45] In the present study, the soil's pH decreased over time. This can be due to consumption of organic carbon of the soil and production of materials by the bacteria. Evidence has indicated a direct association between temperature and activity of microorganisms in such a way that the activity of microorganisms increased through the increase of temperature and vice versa.[46, 47] In this study, temperature also increased over time; this might be due to the growth of bacteria, increase of the microorganisms' activity, and further consumption of hexadecane during an exothermic reaction. However, the amount of dissolved oxygen decreased over time (Figure 5). This could result from the direct relationship between dissolved oxygen and temperature insuch a way that increase of temperature decreases the amount of the slurry dissolved oxygen. One other mechanism might be the increase of the number of microorganisms and their biological activity that cause further dissolved oxygen to be consumed. Juneson and colleagues investigated biodegradation of Bis (2-ethylhexyl) phthalate in the soil by SSBR and came to the conclusion that the dissolved oxygen rate decreased from the zero to the

seventh day.[48] In a co-metabolism study, Giordano and colleagues assessed the biodegradation of lagoon contaminated with PAH by using Sequencing Batch Reactor (SBR) at different detention times (35, 70, 98 days) and along adding anexternal carbon source (Lactose). They reported that the removal rate of PAH was close to 55% at all the detention times.[49] Hence, adding an external carbon source (lactose) didnot have considerable effects on the removal efficiency of PAH. In the present study also, adding an external carbon source (glucose) was not highly effective in removal of hexadecane at 1% concentration (Figure 6). In fact, the total removal rate of hexadecane was only increased by 5% on the second and third days, which might be due to the consumption of glucose by the bacteria and the external carbon source (glucose) being finished. Yet, it should be noted that the total removal rate of hexadecane was increased by 16% on the first day which is 1.4 times higher than the removal rate of hexadecane in the conditions without co-metabolism. Therefore, the effect of co-metabolism was considerable on the first day, but adequate glucose was not used by the bacteria on the second and third days. The study by Giordana and colleagues, revealed that glucose consumption was quite faster than lactose consumption.[49] In the present study, the number of bacteria was 2×10^6, 9×10^7, and 9×10^8 on the first, second, and third days,respectively.This implies that adding the secondary carbon source caused the bacterial growth to increase.

Conclusion

In this study, SSBR was used by application of a bacterial consortium, including *Acinetobacter radioresistens*, *Bacillus subtilis,* and *Pseudomonas aeruginosa,* to remove hexadecane from the soil at different initial concentrations (1-10%). According to the study findings, increase in the initial hexadecane concentration in the soil decreased hexadecane removal. Besides, the best condition for total biodegradation was at the concentration of 1% (10000 mg hexadecane/kg dry soil). In this study, the removal rate of hexadecane reached 45.95% (mg hexadecane/kg dry soil) on the third day. The biodegradation rate of hexadecane was respectively 45.95%, 38.55%, 34.39%, and 32.4% at the concentrations of 1, 4, 7, and 10 percent at the end of the third day. Furthermore, the dissolved oxygen rate decreased following the increase in the microbial activity. In addition, the medium changed much more through the acidic condition, while the conditions never became anaerobic or anoxic. Also, pH did not intolerably decrease by the bacteria. Moreover, adding the external carbon source (glucose) was highly effective in removal of hexadecane on the first day, but this effect decreased in the subsequent days. Overall, the findings of the current study showed that SSBR could be used as an effective method for the soils contaminated with oil products. Also, the secondary carbon source should gradually be added to the reactor in order to increase its efficiency.

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References

1 Volke-Sepúlveda TL, Gutiérrez-Rojas M, Favela-Torres E. Biodegradation of hexadecane in liquid and solid-state fermentations by Aspergillus niger. Bioresour Technol 2003; 87(1): 81-6.

2 Gallegos Martïnez M, Gomez Santos A, Gonzalez Cruz L, Montes de Oca Garcia MA, Yanez Trujillo L, Zermeno Eguia Lis JA, et al. Diagnostic and resulting approaches to restore petroleum-contaminated soil in a Mexican tropical swamp. Water Science and Technology 2000; 42(5-6): 377-84. PubMed PMID: 384.

3 Atlas RM. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev 1981; 45(1): 180-209. PubMed PMID: 239.

4 Bakhshi Nejad B. Isolation, characterization and molecular identification of the bacteria inhabiting the petroleum-contaminated soil [MSc. thesis in genetics]: Tarbiat Modares University, Faculty of Basic Sciences; 2008.

5 Vidali M. Bioremediation. An overview. Pure and Applied Chemistry 2001; 73(7): 1163-72.

6 Pangman JK. Guide to environmental issues: DIANE Publishing; 1995.

7 Anderson R, Rasor E, Van Ryn F. Particle size separation via soil washing to obtain volume reduction. J Hazard Mater 1999; 66(1): 89-98.

8 AKAGI MMMTH. Modeling of Hydrocyclone Circuit on Particle Separation during Soil Washing. Journal of Japan Society on Water Environment 2004; 27(8): 535-40.

9 Shiratori T. Contaminated Soil Remediation. Journal of the Mining and Materials Processing Institute of Japan 2003; 119(8): 441-50.

10 Samaei MR. Combined bioaugmentation and biostimulation to cleanup soil contaminated with hexadecane in slurry bioreactors: Tarbiat Modares University; 2013.

11 Dehghani M, Taatizadeh SB, Samaei MR. Biodegradation of n-Hexadecane in Acinetobacter Radioresistens Liquid Culture. Health Scope 2013; 2(3): 162-7.

12 Hassanshahian M, Ahmadinejad M, Tebyanian H, Kariminik A. Isolation and characterization of alkane degrading bacteria from petroleum reservoir waste water in Iran (Kerman and Tehran provenances). Mar Pollut Bull 2013; 73(1): 300-5.

13 Puskas K, Al-Awadhi N, Abdullah F, Literathy P. Remediation of oil-contaminated sandy soil in a slurry reactor. Environment International 1995; 21(4): 413-21.

14 Cassidy DP, Efendiev S, White DM. A comparison of CSTR and SBR bioslurry reactor performance. Water Res 2000; 34(18): 4333-42. PubMed PMID: 147.

15 Bhandari A, Dove DC, Novak JT. Soil washing and biotreatment of petroleum-contaminated soils. Journal of Environmental Engineering 1994; 120(5): 1151-69.

16 Geerdink M, Kleijntjens R, Loosdrecht Mv, Luyben KCA. Microbial decontamination of polluted soil in a slurry process. Journal of Environmental Engineering 1996; 122(11): 975-82.

17 Nano G, Borroni A, Rota R. Combined slurry and solid-phase bioremediation of diesel contaminated soils. J Hazard Mater 2003; 100(1-3): 79-94. PubMed PMID: 191.

18 Cassidy DP, Hudak AJ. Microorganism selection and biosurfactant production in a continuously and periodically operated bioslurry reactor. J Hazard Mater 2001; 84(2-3): 253-64. PubMed PMID: 244.

19 Khezri SM, Fatemi SH, Poshtegal MK, Hasanlou S. Effect of slurry sequencing batch reactor for bioremediation of TPH contaminated soil. Journal of Food, Agriculture & Environment 2010; 8: 3&4.

20 Hasanlou S. Laboratory-scale bioremediation experiments on diesel and Polycyclic aromatic hydrocarbons contaminated soils. Global Journal of Researches In Engineering 2011; 11(5_B).

21 Timmis KN, McGenity T, Van Der Meer J, De Lorenzo V. Handbook of hydrocarbon and lipid microbiology: Springer Berlin/Heidelberg; 2010.

22 Samaei MR, Mortazavi SB, Bakhshi B, Jonidi Jafari A, editors. Isolation and Characterization of bacteria degrading n-Hexadecane from soil. 2012 International Conference on Biological and Life Science; 2012: IACSIT Press.

23 Cappello S, Santisi S, Calogero R, Hassanshahian M, Yakimov M. Characterisation of oil-degrading bacteria isolated from bilge water. Water, Air, & Soil Pollution 2012; 223(6): 3219-26.

24 Jurelevicius D, Cotta SR, Peixoto R, Rosado AS, Seldin L. Distribution of alkane-degrading bacterial communities in soils from King George Island, Maritime Antarctic. European Journal of Soil Biology 2012; 51: 37-44.

25 Quatrini P, Scaglione G, De Pasquale C, Riela S, Puglia A. Isolation of Gram-positive n-alkane degraders from a hydrocarbon-contaminated Mediterranean shoreline. J Appl Microbiol 2008; 104(1): 251-9.

26 Plangklang P, Reungsang A. Bioaugmentation of carbofuran residues in soil using Burkholderia cepacia PCL3 adsorbed on agricultural residues. International Biodeterioration & Biodegradation 2009; 63(4): 515-22. PubMed PMID: 56.

27 EPA Method 3550c. Ultrasonic Extraction 2007; 17: 1-17.

28 Shojaosadati SA. Industrial biotechnology. Tehran: Center of Scientific Publications; 2010.

29 Samaei MR, Mortazavi SB, Bakhshi B, Jafari AJ. Isolation and Characterization of Bacteria Degrading n-Hexadecane from Soil. International Proceedings of Chemical, Biological & Environmental Engineering 2012; 40.

30 Bernardez L. A rotating disk apparatus for assessing the biodegradation of polycyclic aromatic hydrocarbons transferring from a non-aqueous phase liquid to solutions of surfactant Brij 35. Bioprocess and Biosystems Engineering 2009; 32(3): 415-24. PubMed PMID: 218.

31 Hassanshahian M, Ahmadinejad M, Tebyanian H, Kariminik A. Isolation and characterization of alkane degrading bacteria from petroleum reservoir waste water in Iran (Kerman and Tehran provenances). Marine Pollution Bulletin. 2013.

32 Atlas RM. Microbial hydrocarbon degradation—bioremediation of oil spills. Journal of Chemical Technology and Biotechnology 1991; 52(2): 149-56.

33 Cunha CD, SGF L. GASOLINE BIODEGRADATION IN DIFFERENT SOIL MICROCOSMS. Brazilian Journal of Microbiology 2000; 31: 45-9.

34 Watanabe k, Hamamura N. Molecular and physiological approaches to understanding the ecology os pollutant degradation. Curr Opin Biotechnol 2003; 14(3): 289-95. PubMed PMID: 685.

35 Rahman PK, Gakpe E. Production, characterisation and applications of biosurfactants-Review. 2008.

36 Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P. Biosurfactants: properties, commercial production and application. Current Science 2008; 94(6): 736-47.

37 Vasileva-Tonkova E, Galabova D, Stoimenova E, Lalchev Z. Characterization of bacterial isolates from industrial wastewater according to probable modes of hexadecane uptake. Microbiological Research 2008; 163(4): 481-6.

38 Roy R, Greer CW. Hexadecane mineralization and denitrification in two diesel fuel-contaminated soils. FEMS Microbiology Ecology 2000; 32(1): 17-23. PubMed PMID: 137.

39 Dashti N, Al-Awadhi H, Khanafer M, Abdelghany S, Radwan S. Potential of hexadecane-utilizing soil-microorganisms for growth on hexadecanol, hexadecanal and hexadecanoic acid as sole sources of carbon and energy. Chemosphere 2008; 70(3): 475-9. PubMed PMID: 131.

40 Gomes EB, Silva RF, Rosado AS, Pereira Jr N. Biotreatment of diesel waste by sequencing batch bioreactor operation mode (SBR). International

Biodeterioration & Biodegradation 2010; 64(5): 413-7.

41 Venkata Mohan S, Prasanna D, Purushotham Reddy B, Sarma P. Ex situ bioremediation of pyrene contaminated soil in bio-slurry phase reactor operated in periodic discontinuous batch mode: Influence of bioaugmentation. International Biodeterioration & Biodegradation 2008; 62(2): 162-9.

42 Li H, Zhang Y, Kravchenko I, Xu H, Zhang CG. Dynamic changes in microbial activity and community structure during biodegradation of petroleum compounds: a laboratory experiment. Journal of Environmental Sciences 2007; 19(8): 1003-13.

43 Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. Proc Nati Acad Sci U S A 2006; 103(3): 626-31.

44 Lauber CL, Hamady M, Knight R, Fierer N. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl Environ Microbiol 2009; 75(15): 5111-20.

45 Partovinia A, Naeimpoor F, Hejazi P. Carbon content reduction in a model reluctant clayey soil: Slurry phase n-hexadecane bioremediation. J Hazard Mater 2010; 181(1–3): 133-9.

46 Ratkowsky D, Olley J, McMeekin T, Ball A. Relationship between temperature and growth rate of bacterial cultures. J Bacteriol 1982; 149(1): 1-5.

47 Farrell J, Rose A. Temperature effects on microorganisms. Annu Rev Microbiol 1967; 21(1): 101-20.

48 Juneson C, Ward OP, Singh A. Biodegradation of bis(2-ethylhexyl)phthalate in a soil slurry-sequencing batch reactor. Process Biochemistry 2001; 37(3): 305-13. PubMed PMID: 153.

49 Giordano A, Stante L, Pirozzi F, Cesaro R, Bortone G. Sequencing batch reactor performance treating PAH contaminated lagoon sediments. J Hazard Mater 2005; 119(1–3): 159-66.